SI Appendix

SI Appendix Methods

Cell cultures. *Human monocyte-derived macrophages (HMDM*). Studies using cells isolated from healthy donors were approved by the Colorado Multiple Investigation Review Board. HMDM were differentiated from peripheral blood mononuclear cell (PBMCs). Venous blood from healthy consenting donors was drawn into lithium heparin containing tubes and PBMCs were isolated using centrifugation over Ficoll-Hypaque cushions as previously described (48). Cells were suspended in RPMI (Corning, Manassas, VA) supplemented with 1% penicillin/streptomycin (P/S) at $5x10^6$ cell/ml and seeded $0.5x10^6$/well in 96-flat bottom well plates in a total of 200 µl RPMI for 2 hours. Non-adherent cells were discarded and the adherent monocytes incubated for 6 days in RPMI supplemented with 10% fetal bovine serum (FBS), 1% P/S and human GM-CSF (5 ng/ml) (R&D Systems, Minneapolis, MN). On days 3 and 5, the medium was refreshed.

Mouse macrophages. J774A.1 cell line was cultured in Dulbecco's modified Eagle's medium (Corning, Manassas, VA) supplemented with 10% FBS and 1% P/S at 37°C with 5% CO_2. Cells were seeded $1x10^5$/well in 96-flat bottom well plates and cultured in 200 µl overnight before stimulation.

U937 cells. U937 cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and were grown in RPMI supplemented with 10% fetal calf serum (FCS) and 2 mM L-glutamine.

THP-1 cells. THP-1 cells were cultured in RPMI 10% FBS and 1% P/S and seeded at the concentration of $2.5x10^5$/well in 96-flat bottom well plates and cultured in 200µl overnight before stimulation.

Human blood neutrophils. Neutrophils were isolated from the freshly obtained peripheral blood of healthy donors using Polymorphoprep™ (Axis-Shield PoC A(49)S, Oslo, Norway) as described previously (49). This protocol routinely yields more than 97% cell purity as assessed by flow cytometry and Wrights-Giemsa stain (Sigma-Aldrich).

CAPS patients. Blood samples from two CAPS patients positive for the mutation of the *nlrp3* gene were taken after informed consent by patients or their legal guardian and as approved by the "G Gaslini" Ethical Board.

Inflammasome stimulation. Cells were stimulated with 1 µg/ml LPS (*Escherichia coli* (055:B5, Sigma-Aldrich, St. Louis, MO) for 4 hours and either nigericin (10 µM, InvivoGen, San Diego, CA) or ATP (5 mM, Sigma-Aldrich) was added for 40 minutes or 1 hour. OLT1177 was added to the cells either 30 minutes following LPS stimulation or at the same time as ATP or nigericin.

For activation of NLRC4, J774A.1 cells were stimulated in FBS free media with LPS (1 μg/ml) for 2 hours and Flagellin (3.75 μg/ml, isolated from *Salmonella typhimurium,* Enzo Life Sciences, Farmingdale, NY) was added for overnight incubation before the supernatants were harvested. Similarly, for AIM2 activation, cells were stimulated with LPS for 2 hours, and 5 μg/ml of deoxyadenylic-deoxythymidylic acid (PolydA:dT, InvivoGen) was added for overnight incubation. Supernatants were obtained for assay.

To induce non-canonical NLRP3 inflammasome activation, THP-1 cells were primed with 100 ng/ml Pam3CSK4 (InvivoGen) for 4 hours. The medium was removed, replaced with serum-free medium containing OLT1177 for 1 hour, then the cells were transfected with LPS (2 μg/ml) using the delivery agent PULSin (Polyplus Transfection, NY) for an additional 4 hours.

Monocytes from CAPS patients were stimulated with LPS (100 ng/ml) for 18 hours. OLT1177 was added to media 30 minutes following LPS.

Caspase-1 activity assay. Caspase-1 activity was determined in J774A.1 cell lysates by cleavage of a fluorogenic substrate, as previously described (50). Briefly, after removal of supernatants, the cells were lysed using RIPA buffer (Sigma-Aldrich) containing a mixture of protease inhibitors (Roche, Indianapolis, IN) and centrifuged at 13,000g for 20 minutes at 4°C. The cell pellets were suspended in caspase-1 reaction buffer (31% sucrose, 3.1% HEPES and 0.31% CHAPS; Sigma-Aldrich) and incubated in the dark at 30°C with the actifluorescence substrate (A2452; Sigma-Aldrich) for 80 minutes to allow the progression of the reaction. Fluorescence was reported as arbitrary fluorescence units generated by 1 μg of sample per minute (fluorescence/μg/min) and calculated as fold change compared to LPS/NIG. Caspase-1 activity in human neutrophils was determined using Caspase 1 Inhibitor Drug Detection assay as recommended by the manufacturer (Abcam, Cambridge, UK). In brief, neutrophils were incubated with LPS/ATP with and without 10 or 50 μM of OLT1177. Cells were lysed in HEPES buffer with Triton X-100 (Sigma-Aldrich) and lysates were mixed with reaction buffer containing the fluorogenic substrate, YVAD (50 μM final concentration). Fluorescence was determined at 400 nm excitation and 505 nm emission wavelengths on Tecan infinite M200 plate reader (Männedorf, Switzerland). Active caspase-1 and synthetic caspase-1 inhibitor zVAD FMK were used as a positive control for the assay.

Cytokine and cell viability measurement. Cytokines in supernatants and cell lysates were measured by specific ELISA according to the manufacturer's instructions (DuoSet, R&D Systems, Minneapolis, MN). Lactate dehydrogenase (LDH) release was measured in supernatants using the LDH assay (Biovision, Mountain View, CA). Because of the large

variation in primary cells as well cell lines the data are transformed to percent change for each concentration of OLT1177. For example in LPS stimulated cultures each mean value in pg/ml (range of the stimulation is indicated in each figure legend) is set at 100%. For each value of OLT1177 concentration percent change is calculate. The mean per change is depicted for the entire dose response.

Flow Cytometry. C57BL/6 mice from Charles River were treated with five intraperitoneal doses of OLT1177 (200 mg/kg) in 200 µl every 12 hours or matching volume of vehicle (saline). After sacrifice, the spleens were removed, homogenized using 40 µm cell strainers, washed and suspended in RPMI at $5x10^6$/ml. An aliquot of cells was immediately fixed with freshly prepared 1% paraformaldehyde. The remaining cells were seeded at $0.5x10^6$/ml in 96-round bottom well plates in RPMI with 10% FBS and treated with LPS 2.5 µg/ml for 30 minutes followed by fixation. For membrane staining, anti-CD3 BV421 (Biolegend, San Diego, CA) and anti-CD11b PerCP-Cy5.5 (Biolegend) with the isotype controls (eBioscience or Bio-Techne) were incubated for 30 min at 4 °C. For intracellular staining, cells were fixed and permeablized as recommended by the manufacturer's protocol (InvitroGen). Staining was performed in four separate panels with phosphoNFκB-AlexaFluor 647 (Cell Signaling, Danvers, MA) and NFκB-PE (Cell Signaling); phosphoIκB-eFluor 660 (eBioscience, San Diego, CA) and IκB-PE (eBioscience); phosphoIKK-PE (Cell Signaling) and IKK-BV605 (Novus Biologicals, custom conjugate); phosphoIRAK4-PE (Cell Signaling) and IRAK4 AlexaFluor 647 (Novus Biologicals, custom conjugate) for 1 hour at 4°C. Cells were then washed twice and resuspended in fix buffer for flow analysis.
Cells were analyzed using an LSR-II flow cytometer (BD Immunocytometry Systems). Between $0.1 x10^6$ and $1 x10^6$ events were collected. Electronic compensation was performed with antibody capture beads (BD Bioscience) stained separately with individual antibodies used in the test samples. Lymphocytes, and myeloid cells were gated based on their forward and side scatter profile, and $CD3^+CD11b^-$ T cells or $CD3^-CD11b^+$ myeloid cells were enumerated for total and phosphorylated intracellular targets. The data were analyzed using FlowJo Software (Treestar), and biexponential scaling was used.

NLRP3 ATPase activity. Human recombinant NLRP3 (0.105µg; Novus Biologicals, Littleton, CO) was incubated with OLT1177, Bay 11-7082 (BAY, InvivoGen) and 3,4-methylenedioxy-β-nitrostyrene (MNS, EMD Millipore, Massachusetts) in the reaction buffer (20 mm Tris-HCl, pH 7.8, 133 mm NaCl, 20 mm MgCl2, 3mm KCl, 0.56 mm EDTA, 0.5% DMSO) for 15 min at 37°C. Ultra pure ATP (250µM) was added for 40 min at 37°C. The hydrolysis of ATP by NLRP3 was determined by ADP-Glo Kinase Assay (Promega, Madison, MI, USA) according to

the manufacturer's protocol.

Immunoprecipitation. J774A.1 cells were stimulated with LPS (1µg/ml) for 4h and nigericin (10 µM) for 40 min. OLT1177 was added 30 minutes after LPS. Cells were lysed with cold NP-40 lysing buffer (1% NP-40, 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl) supplemented with Roche Complete Protease Inhibitor. The cell lysates were incubated overnight at 4°C with primary antibody for NLRP3 (Cryo-2, AdipoGen San Diego, CA) and Protein G Mag Sepharose (GE Healthcare). ASC was determined by western blotting (D2W8U, (Cell Signaling).

Western blotting. Cells were lysed using RIPA buffer supplemented with protease inhibitors (Roche), centrifuged at 13,000g for 20 min at 4 °C and the supernatants were obtained. Protein concentration was determined in the clarified supernatant using Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Supernatants from stimulated J774A.1 cells were concentrated using StrataClean resin (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. Proteins were electrophoresed on Mini-PROTEAN TGX 4−20% gels (Bio-Rad Laboratories) and transferred to nitrocellulose 0.2µM (GE Water & Process Technologies). Membranes were blocked in 5% dried milk in PBS-T 0.5% for 1 hour at room temperature. Primary antibodies for caspase-1 1:200 (sc-514 Santa Cruz Biotechnology, Dallas, TX, USA), and IL-1β 1:1000 (AF-401 R&D Systems) were used in combination with peroxidase-conjugated secondary antibodies and chemiluminescence to detect the protein concentration. A primary antibody against β-actin (Santa Cruz Biotechnology) was used to assess protein loading.

Real-time PCR. RNA was isolated according to the manufacturer's protocol (Qiagen) and synthesized into cDNA using SuperScript III First-Strand (Thermo Fisher Scientific). Quantitative PCR (qPCR) was performed on cDNA using Power SYBR Green PCR master mix (Thermo Fisher Scientific) on Biorad CFX96 Real time system. Gene expression was carried for the following mRNAs: *nlrp3*, *asc, caspase1, il1b* and *il18* with *gapdh* used as reference gene.

Phosphoarray assay. HMDM were stimulated with LPS/NIG in presence and absence of OLT1177 (10µM) as previously described. Phosphorylation kinases profile was measured in the cell lysate using Proteome Profiler (R&D Systems, Inc.) according to the manufacturer's protocol.

Metabolomics analysis. Quadriceps muscle (10 mg) from mice challenged with LPS and treated with vehicle or OLT1177 were homogenized in 1 ml of ice-cold lysing buffer (methanol: acetonitrile: water 5:3:2), vortexed for 30 min at 4ºC and then centrifuged at 15000g for 10

minutes at 4°C. After discarding protein pellets, 20 μl of the water/methanol soluble fractions were assayed using an ultra-high performance liquid chromatography (UHPLC) (Vanquish, Themo Fisher). UHPLC employed a C18 reversed phase column (phase A: water, 0.1% formic acid; B: acetonitrile, 0.1% formic acid - Phenomenex, Torrance, CA). UHPLC was coupled on line with a high resolution quadrupole Orbitrap instrument operated in either polarity modes (QExactive, Thermo Fisher) at 70,000 resolution (at 200 m/z). Maven software (Princeton), KEGG pathway database, and an in-house validated standard library (>800 compounds; Sigma-Aldrich; IROATech, Bolton, MA) were used to validate metabolite assignment and peak integration for relative quantitation. Integrated peak areas were exported into Excel (Microsoft, Redmond, CA) and elaborated for statistical analysis (T-Test, ANOVA).

Heterologous expression of human P2X7R in oocytes and TEVC measurements. *Xenopus laevis* ocytes were purchased from Ecocyte Bioscience (Castrop-Rauxel, Germany), derived from adult female South African Clawed Frogs (*Xenopus laevis*; Xenopus-Express, Le Bourg, France) and prepared as previously described (47). Oocytes of stages V and VI were injected with cRNA encoding for P2X7R (0.35 ng per oocyte) using a microinjector (Nanoject, Drummond Scientific, Broomall, USA). P2X7R cRNA was kindly provided by G. Schmalzing (RWTH Aachen University, Department of Molecular Pharmacology, Aachen, Germany). The injection volume was 50 nl. Controls for two-electrode voltage-clamp (TEVC) experiments were performed with oocytes that were injected with 50 nl of nuclease-free water. After an incubation time of 1-2 days, the transmembrane currents (I_M) of water- or RNA-injected oocytes were recorded by the TEVC technique at room temperature. Oocytes were placed in a perfusion chamber and perfused (gravity driven) with a Ca^{2+}-free frog Ringer's solution containing (mM): 100 NaCl, 2.5 KCl, 5 HEPES and 0.1 flufenamic acid (pH 7.4). All chemicals used for preparation of frog Ringer's solution were purchased from Fluka, except for HEPES and flufenamic acid (Sigma-Aldrich). Intracellular microelectrodes were pulled from borosilicate glass capillaries and filled with 1M KCl solution. The membrane voltage was clamped to −40 mV using a TEVC amplifier (Warner Instruments, Hamden, CT), I_M were low-pass filtered at 1000 Hertz (Frequency Devices 902, Haverhill, MA) and recorded via a strip chart recorder (Kipp & Zonen, Delft, Netherlands). A stock solution of 100 mM BzATP (Jena Bioscience) was prepared and dissolved in frog Ringer's solution to a working solution of 10 μM BzATP. BzATP was applied for 2 min in presence or absence of OLT1177 (100 μM and 0.1 nM) and BzATP-induced changes of I_M were monitored. In all experimental groups, measurements were performed on oocytes from at least two different *Xenopus laevis* frogs.

Human Safety Study. The clinical trial was a single-center, placebo-controlled, sequential group, dose escalation study of the safety and PK of 3 dosages of OLT1177 Caps in 35 healthy male and female subjects aged 18 to 60 years. OLT1177 Caps were administered orally at doses of 100, 300 and 1,000 mg OLT1177 in single-dose and multiple-dose regimens. In 18 healthy subjects in 3 dose escalating cohorts were randomized to receive a single dose of the investigational drug (5 subjects received OLT1177 and 1 subject placebo). Upon completion, a new subject population of 17 subjects in 3 dose escalating cohorts was randomized. Subjects received a total of 8 doses over 8 consecutive days given once per day and were followed for up to 30 days for safety assessment. Safety and tolerability were assessed by physical examination and measurement of vital signs, ECGs and clinical laboratory tests (e.g., chemistry, hematology, lipid profile, coagulation factor and urinalysis). Adverse events and concomitant medications were also recorded.

Pharmacokinetic analysis

The pharmacokinetic (PK) parameters of OLT1177 and area under the concentration time curve (AUC) were used to characterize systemic drug exposure. The following Day 1 PK parameters were calculated for all cohorts: $AUC_{0\text{-}t}$, $AUC_{0\text{-}inf}$, $AUC_{0\text{-}24}$, Residual area, C_{max}, T_{max}, K_{el}, $T_{1/2\ el}$, Cl/F, R_{Kel}, MRT and V_d/F. PK analyses were performed on plasma concentrations of OLT1177 using Pharsight® Knowledgebase ServerTM and WinNonlin® version 5.3 (Pharsight Corp., Mountain View, CA, USA). Descriptive statistics (n, arithmetic mean, SD, coefficient of variation, median, minimum, maximum and geometric mean) of the plasma concentrations versus time as well as the PK parameters were summarized for each cohort. PK parameters were calculated using standard non-compartmental methods and actual times. All concentration values below the lower limit of quantitation (2 ng/ml) were set to zero for calculation purposes. Samples with no reportable value occurring prior to the first dosing were replaced by “0.00” otherwise they were set to missing for tabulation, graphical representation and calculation purposes. Inferential statistical analyses were interpreted in an exploratory sense only at an alpha level of 5% for statistical significance.

Statistical Analysis. Significance of differences was evaluated with Student’s two-tail T-test using GraphPad Prism (GraphPad Software Inc, La Jolla, CA). The mean percentage change for each concentration of OLT1177 was calculated for each experiment. The data shown represent the mean percentage change each all of the experiment of each condition unless specified. Statistical significance was set at $P<0.05$.

SI Appendix References

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48. Netea MG, *et al.* (2009) Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. *Blood* 113(10):2324-2335.
49. Al-Omari M, *et al.* (2011) Acute-phase protein alpha1-antitrypsin inhibits neutrophil calpain I and induces random migration. *Mol Med* 17(9-10):865-874.
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SI Appendix Tables

Parameter (units)	OLT1177 100 mg (N=5)			OLT1177 300 mg (N=5)			OLT1177 1000 mg (N=5)		
	Mean	**SD**	**CV%**	**Mean**	**SD**	**CV%**	**Mean**	**SD**	**CV%**
$AUC_{0\text{-}t}$ (h·ng/ml)	76457.07	12086.84	15.81	324650.71	94161.49	29	918963.5	168977.72	18.39
$AUC_{0\text{-}inf}$ (h·ng/ml)	76998.33	12060.14	15.66	327323.86	96496.3	29.48	930498.46	178427.88	19.18
$AUC_{0\text{-}24}$ (h·ng/ml)	40121.19	8013.16	19.97	157378.19	34587.51	21.98	464809.56	69517.24	14.96
Residual area (%)	0.71	0.62	87.08	0.74	0.66	89.1	1.12	1.23	109.71
C_{max} (ng/ml)	2700	635	23.5	9800	2220	22.6	32000	9130	28.6
$T_{½\,el}$ (h)	23.01	4.26	18.52	22.8	4.99	21.9	24.15	6.92	28.65
K_{el} (/h)	0.0309	0.0054	17.6244	0.0317	0.0075	23.6393	0.0307	0.009	29.3103
Cl/F (L/h)	1.32	0.21	15.77	0.97	0.24	24.91	1.11	0.21	18.99
V_d /F (L)	43.95	10.29	23.41	31.86	10.32	32.4	37.63	9.25	24.58
Cl/F (L/h/kg)	0.018	0.003	15.31	0.013	0.002	18.44	0.014	0.004	30.75
V_d /F (L/kg)	0.585	0.031	5.37	0.426	0.066	15.47	0.473	0.05	10.58
MRT (h)	30.49	5.16	16.92	30.47	8.14	26.72	32.51	10.82	33.27
R_{Kel}	1.94	0.25	12.6	1.93	0.29	14.79	2.01	0.4	19.82
Parameter (units)	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**
T_{max} (h)	2	0.5	3.5	1.5	1	2.5	1.5	1	3.5

SI Appendix Table S1. Summary of pharmacokinetic parameters for OLT1177 after a single dose.

Parameter (units)	OLT1177 100 mg				OLT1177 300 mg*				OLT1177 1000 mg			
	N	Mean	SD	CV%	N	Mean	SD	CV%	N	Mean	SD	CV%
$AUC_{0-\tau}$ (h·ng/ml)	5	70288.75	13596.29	19.34	3	260409.24	42022.67	16.14	5	723127.44	252327.03	34.89
C_{max} (ng/ml)	5	4800	1360	28.4	4	15800	3670	23.3	5	41400	10800	26.1
C_{min} (ng/ml)	5	1710	246	14.4	4	6820	1640	24	5	18700	6880	36.8
C_{ave} (ng/ml)	5	2930	567	19.3	3	10900	1750	16.1	5	30100	10500	34.9
$T_{½ el}$ (h)	5	16.01	4.45	27.8	3	21.34	1.52	7.11	5	24.72	12.38	50.07
% (FI) (%)	5	102.79	28.39	27.62	3	83.55	12.26	14.68	5	77.72	13.52	17.39
Cl_{ss} /F (L/h)	5	1.47	0.339	23	3	1.17	0.209	17.8	5	1.49	0.402	26.9
V_d /F (L)	5	35.22	16.14	45.81	3	36.19	7.05	19.47	5	48.56	12.89	26.55
Cl_{ss} /F (L/h/kg)	5	0.022	0.005	21.63	3	0.014	0.003	21.37	5	0.017	0.004	21.39
V_d /F (L/kg)	5	0.491	0.043	8.84	3	0.449	0.127	28.24	5	0.586	0.196	33.52
R_{AUC}	5	1.69	0.29	17.2	3	1.75	0.09	4.89	5	1.93	0.1	5.21
Parameter (units)	**N**	**Median**	**Min**	**Max**	**N**	**Median**	**Min**	**Max**	**N**	**Median**	**Min**	**Max**
T_{max} (h)	5	1	1	3	4	1	1	3	5	2	1	3.5

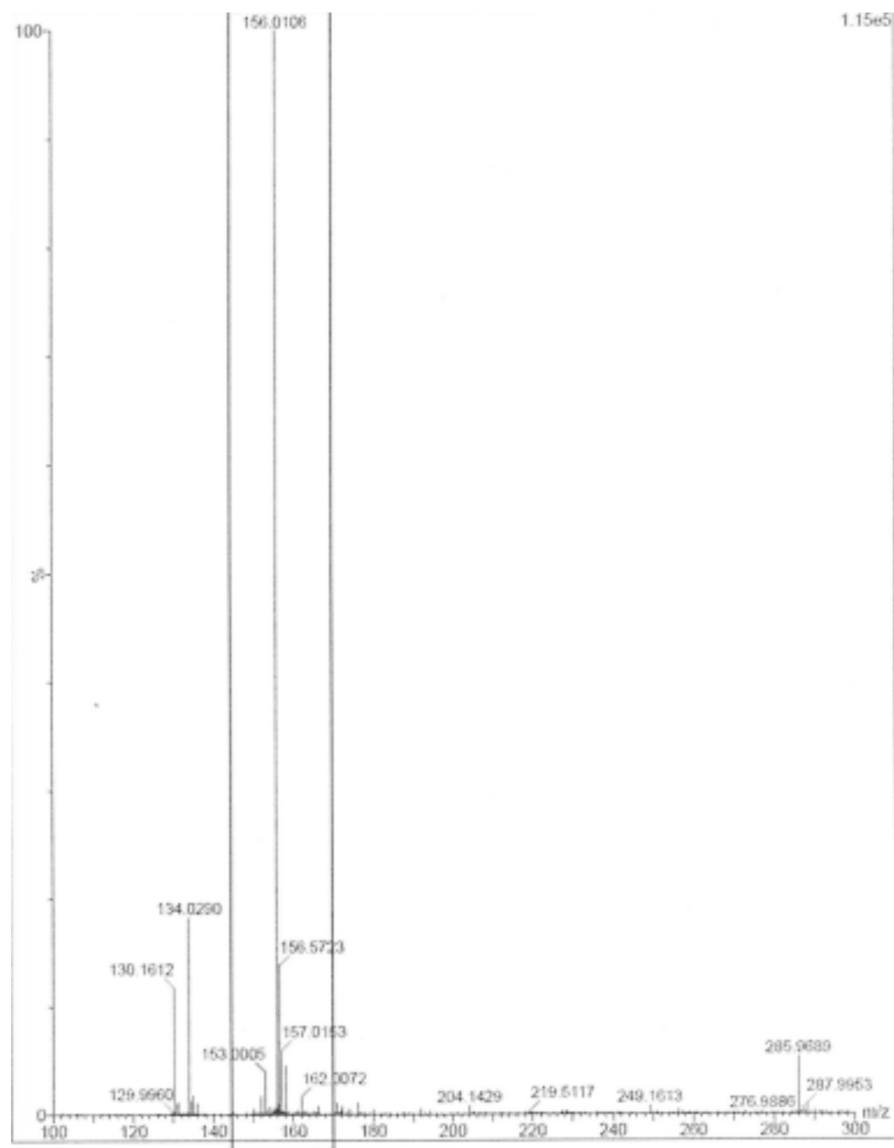
* The elimination rate constant could not be calculated for Subject 001-036, therefore, all PK parameters calculated by extrapolation using K_{el} are not presented (N=3).

SI Appendix Table S2. Summary of pharmacokinetic parameters for OLT1177 after repeated dosing.

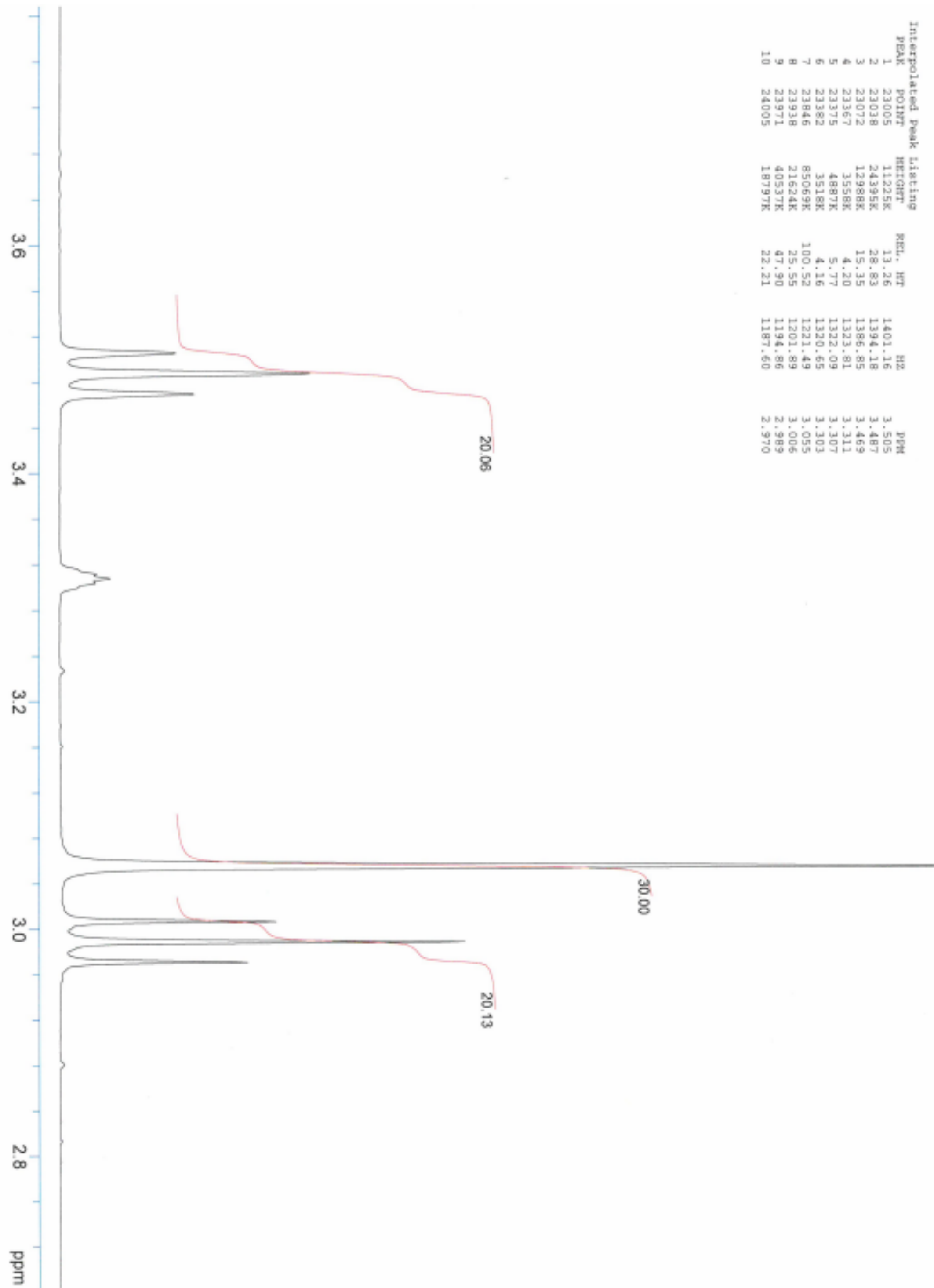
Treatment	Subject ID	Event Description System Organ Class Preferred Term	Start Date Relative to First Dose	Severity	Relationship to Treatment	Action Taken	Outcome
Single Dose Administration							
Placebo	001-001	Nervous System Disorders Headache	Day 13	Moderate	Unrelated	Medication	Resolved
OLT1177 Caps 100 mg Single dose	001-002	Skin and Subcutaneous Tissue Disorders Eczema	Day 13	Mild	Unrelated	None	Resolved
OLT1177 Caps 300 mg Single dose	001-008	Skin and Subcutaneous Tissue Disorders Dermatitis contact	Day 1	Mild	Unrelated	Medication	Resolved
Single Dose Administration with Food Effect							
OLT1177 Caps 1000 mg Multiple doses	001-016	Nervous system disorders Migraine	Day 2	Moderate	Unrelated	Medication	Resolved
OLT1177 Caps 1000 mg Multiple doses	001-023	Musculoskeletal and Connective Tissue Disorders Back pain	Day 9	Mild	Unrelated	Medication	Resolved
Multiple Dose Administration							
OLT1177 Caps 100 mg Multiple doses	001-024	Gastrointestinal Disorders Diarrhoea	Day 3	Mild	Unrelated	None	Resolved
OLT1177 Caps 100 mg Multiple doses	001-025	Nervous System Disorders Headache	Day 1	Moderate	Unrelated	Medication	Resolved

SI Appendix Table S3. Summary of treatment-emergent adverse events.

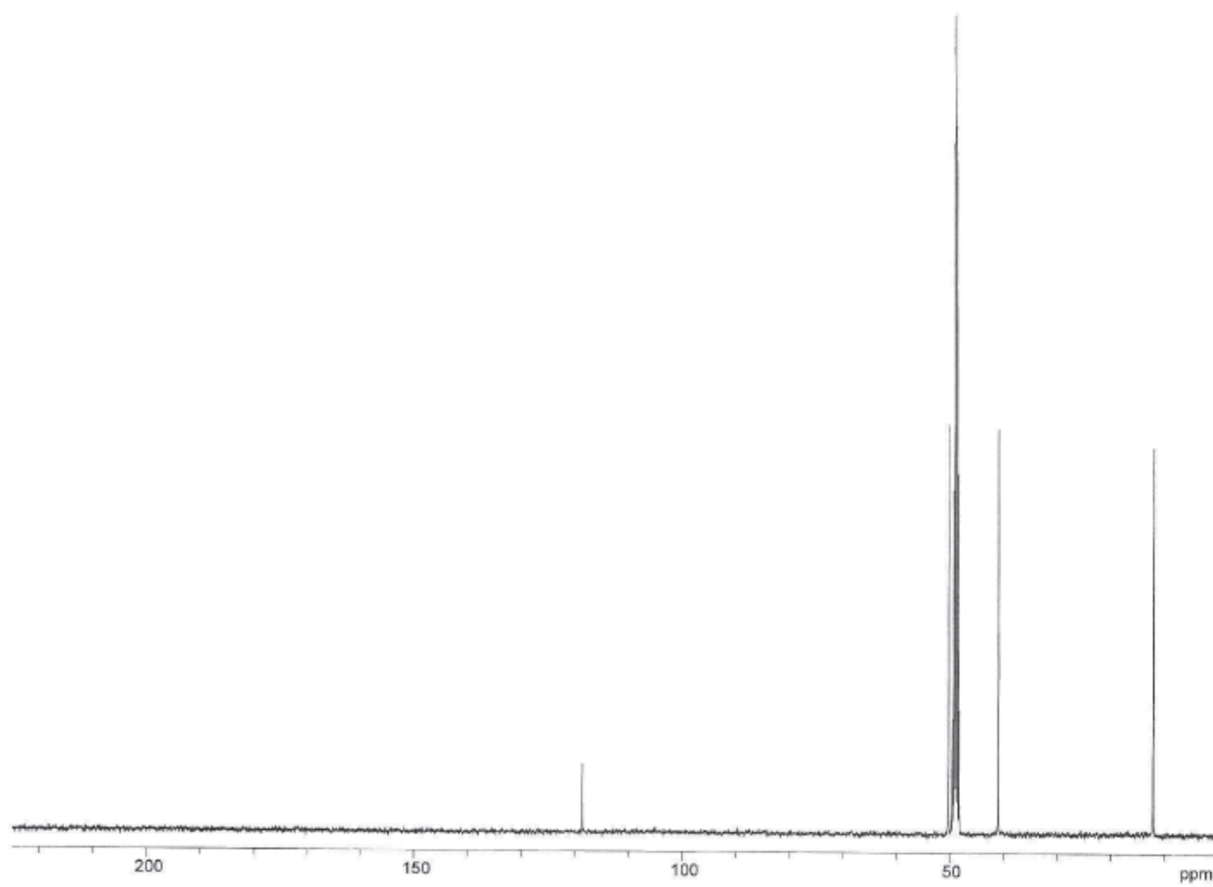
SI Appendix Figures and Figures legend



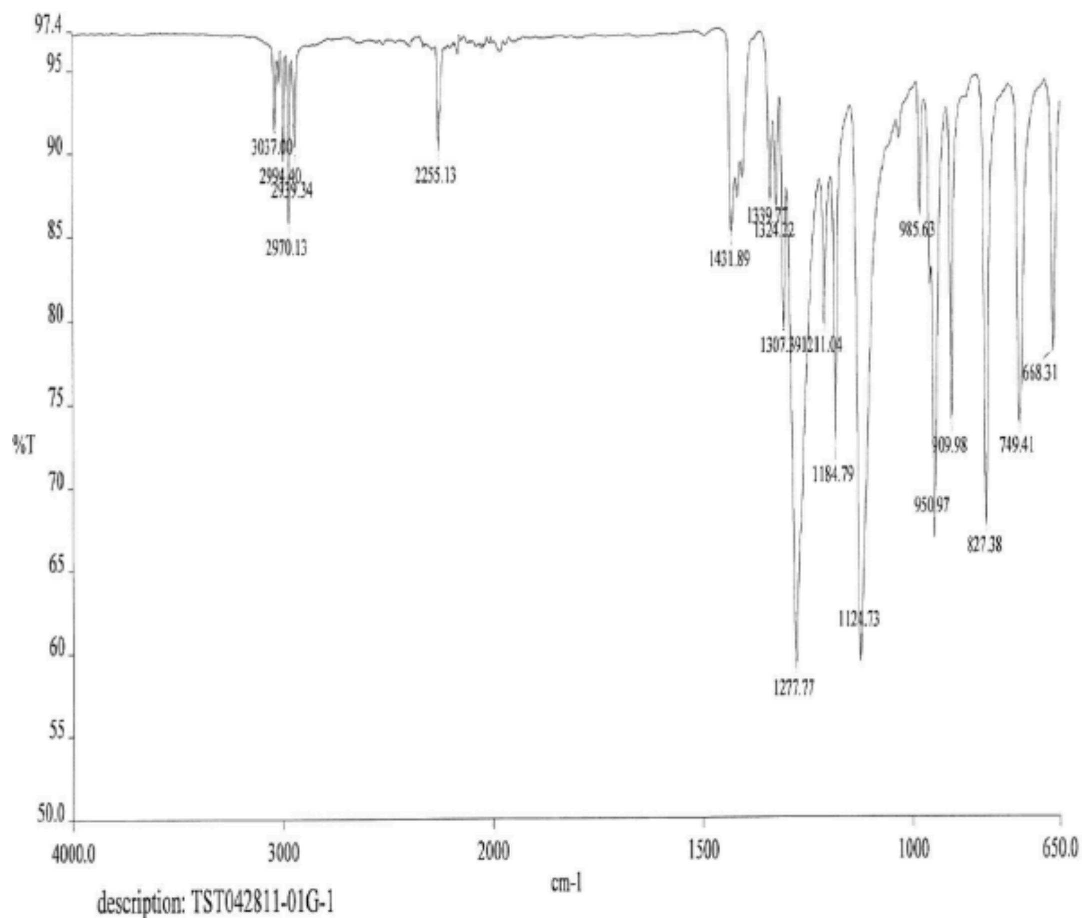
SI Appendix Fig. S1. OLT1177 mass spectrum. High resolution mass spectrometry was performed on OLT1177 using LC-MS. A sample was prepared at a concentration of 2 mg/ml in methanol. The principal fragment was determined to have a mass of 156 g/mol and corresponds to the drug substance (133 g/mol) as the sodium adduct (+23 g/mol). These data confirm that the drug substance has the expected mass.



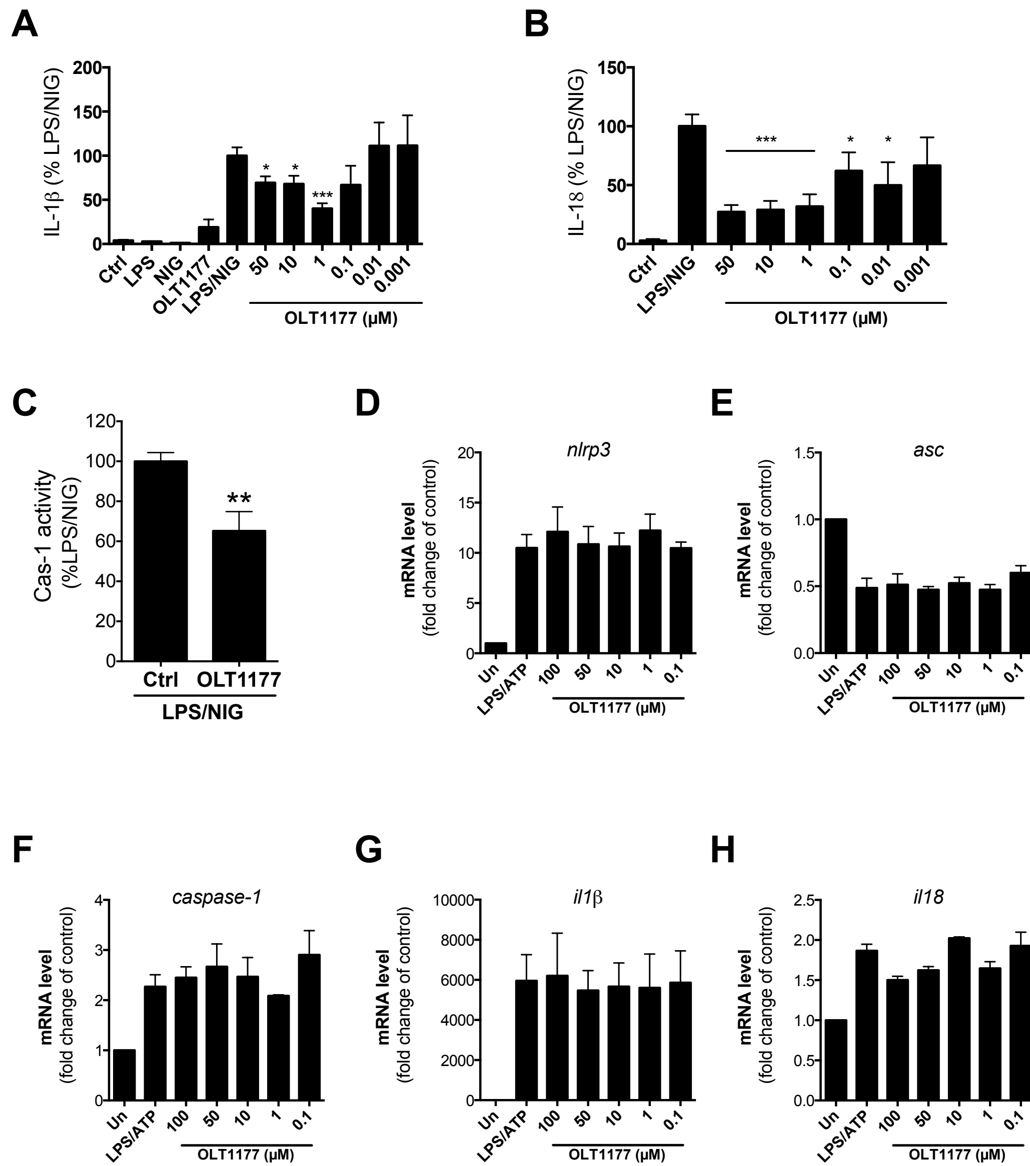
SI Appendix Fig. S2. OLT1177 ^{1}H-NMR spectrum. A ^{1}H-NMR spectrum was obtained for OLT1177 in deuterated methanol (CD_3OD) using a 400 MHz instrument.



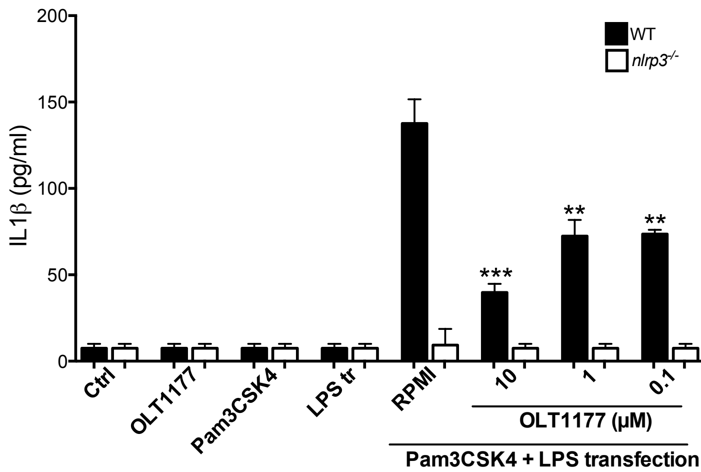
SI Appendix Fig. S3. OLT1177 ^{13}C-NMR spectrum. A ^{13}C-NMR spectrum was obtained for OLT1177 in CD3OD using a 100 MHz instrument.



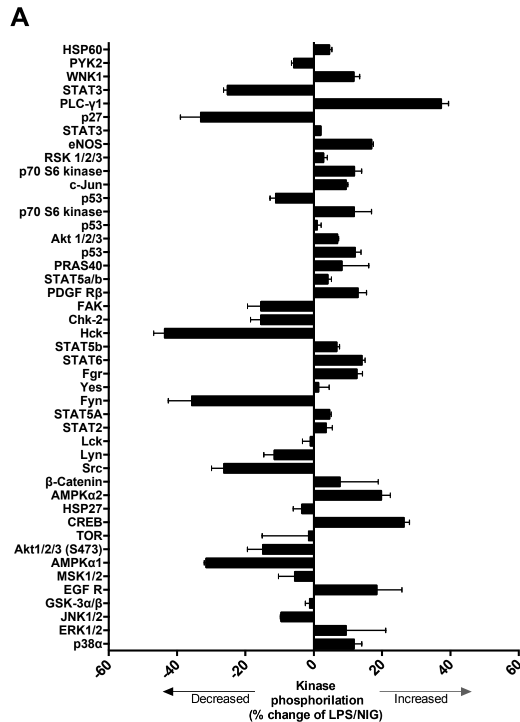
SI Appendix Fig. S4. OLT1177 Fourier Transform Infrared Spectroscopy (FTIR) spectrum. An FTIR spectrum was obtained by introducing OLT1177 into a Fourier transform infrared spectrophotometer equipped with a spectral reflectance interface.

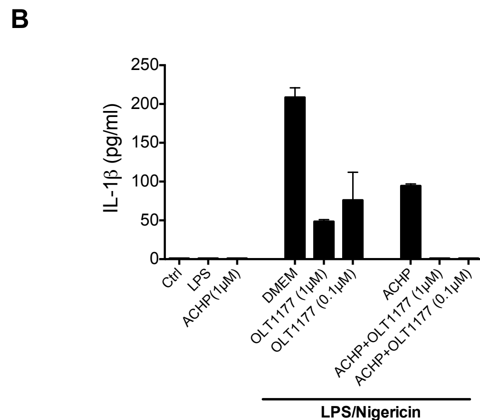


SI Appendix Fig. S5. OLT1177 reduces NLRP3 inflammasome activation in HMDM but has no effect on gene expression. (*A,B*) Mean ±SEM percent change of secreted IL-1β (range 100-2201 pg/ml) (*A*) and IL-18 (range 70-1282 pg/ml) (*B*) in HMDM from 6 healthy donors stimulated with LPS followed by nigericin (NIG) in the presence of increasing concentrations of OLT1177. (*C*) Mean ±SEM of caspase-1 activity in J774A.1 cell lysates following LPS and NIG stimulation in presence of OLT1177 (50 µM). Percent change are calculated as described in the Methods. The black bars represent the percent of IL-1β that is still expressed after treatment. (*D-H*) Fold change of mRNA levels from J774A.1 cells stimulated with LPS and ATP in presence or absence of OLT1177 (100 to 0.1 µM). Data from J774A.1 cells are expressed as mean ±SEM of 3 independent experiments. ***: $P<0.001$, **: $P<0.01$, *: $P<0.05$.

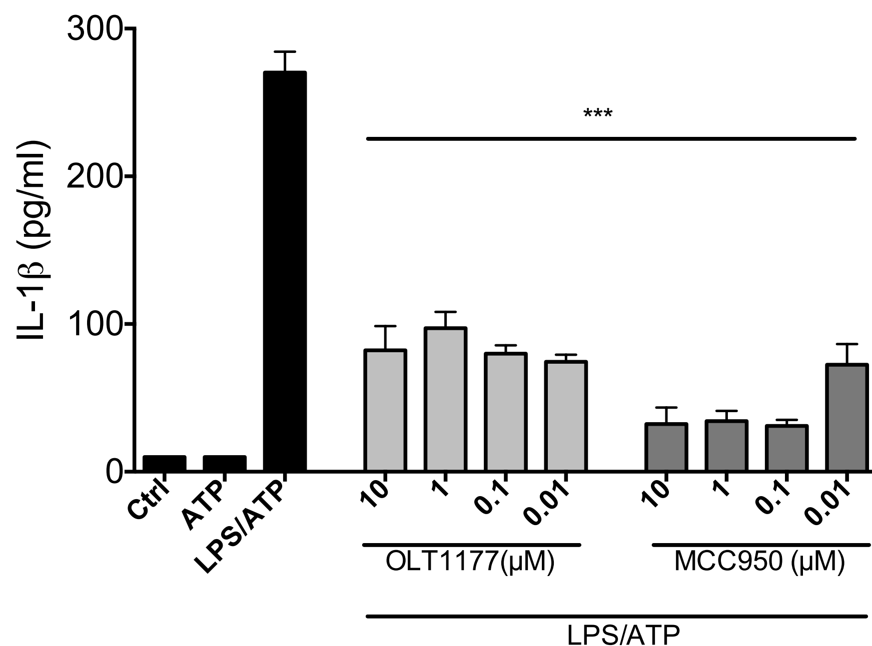


SI Appendix Fig. S6. OLT1177 reduces IL-1β release following non-canonical NLRP3 inflammasome activation. Mean ±SEM of secreted mature IL-1β from wild type and NLRP3 knock out (*nlrp3*$^{-/-}$) THP-1 cells stimulated with Pam3CSK4 and transfected with LPS to induce non-canonical NLRP3 inflammasome in the presence of OLT1177. ***P<0.001, **P<0.01. Data are expressed as mean ±SEM of 3 independent experiments.

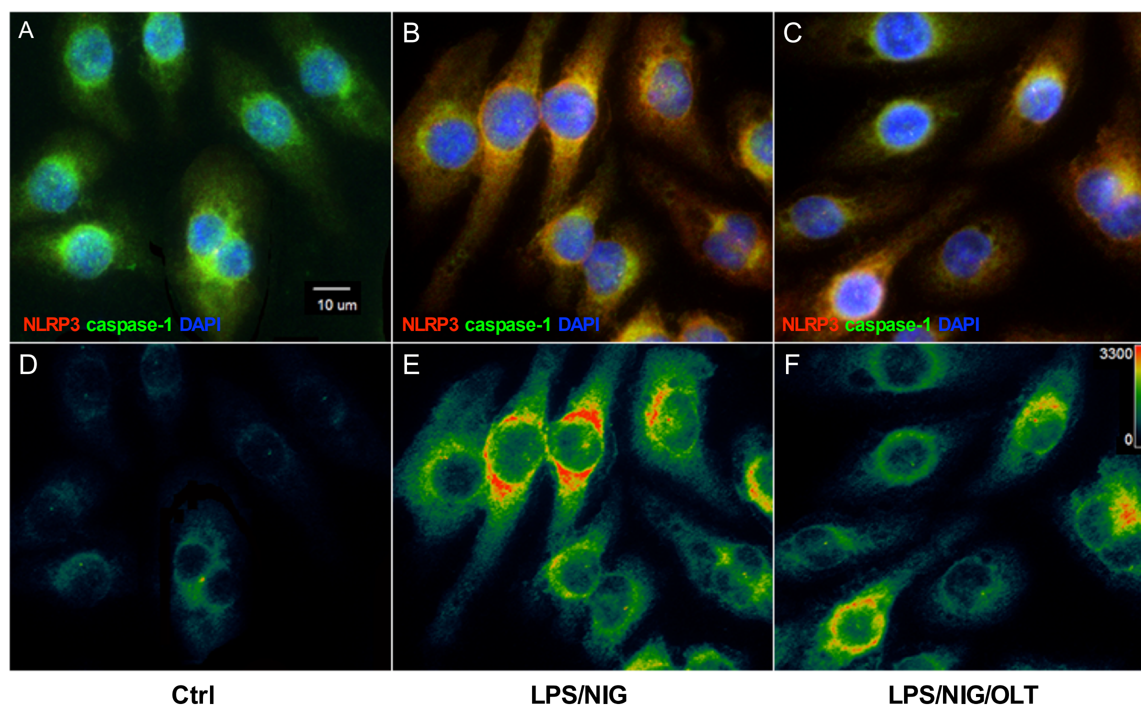




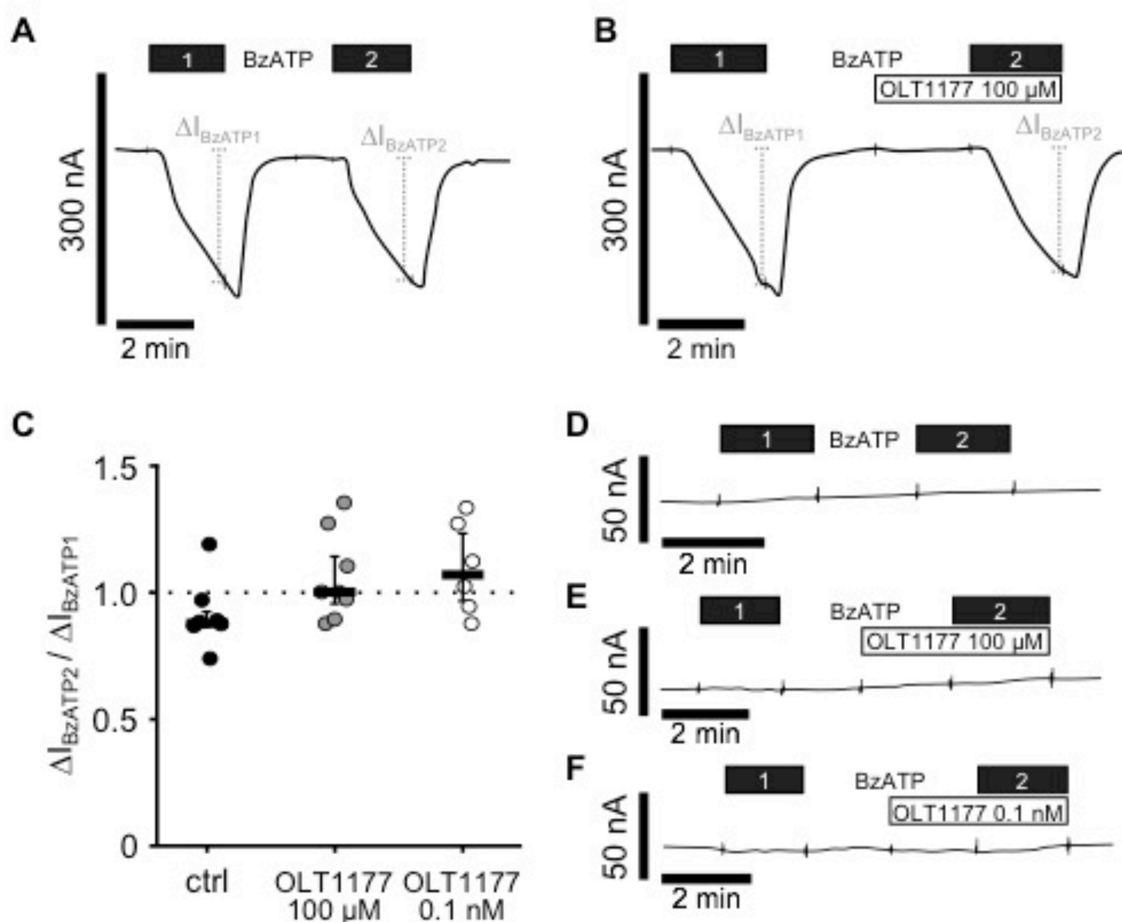
SI Appendix Fig. S7. OLT1177 modulates kinases post-translational modification and has no effect on the priming phase. (*A*) Representative kinase phospho-array for cytosolic non-receptor kinases and other signaling molecules in HMDM derived from one healthy donor. Results are expressed as percent change of signal intensity (AU) of stimulated control (LPS/NIG). (*B*) Mean ±SEM of secreted IL-1β from murine macrophage cell line J774A.1 stimulated with LPS and nigericin in presence of IκB kinase (IKK) inhibitors 2-Amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-(4-piperidinyl)-3-pyridinecarbonitrile (ACHP) (*B*) and OLT1177.



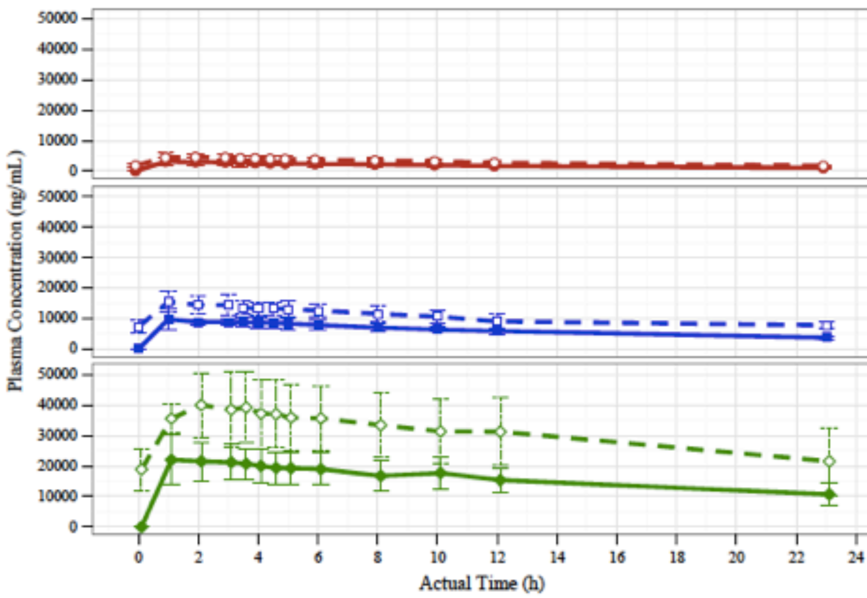
SI Appendix Fig. S8. Comparison between OLT1177 and MCC950 in IL-1β release in J774A.1 following LPS/ATP stimulation. ***P<0.001.



SI Appendix Fig. S9. (*A)* Immunofluorescent staining for caspase-1 (diffuse shown in green in control) in J774A.1 cells. After 4 hours with LPS followed by NIG, NLRP3 (in red) can be observed in the merge image (*B*). As shown in *C*, OLT1177 (10 µM) reveals reduced overlap. (*D-F*) The same cells using FRET analysis under the same conditions reveals the close association of caspase-1 and NLRP3. In the presence of OLT1177 (10 µM), there is reduced association. Representative images of three independent experiments.



SI Appendix Fig. S10. OLT1177 has no effect on BzATP-induced P2X7R activation. (*A, B*) Two-electrode voltage-clamp (TEVC) measurements were performed on human P2X7 receptor (hP2X7R) heterologously expressed in *Xenopus laevis* oocytes. (*A*) Control experiments (ctrl; N=7): hP2X7R agonist BzATP (10 µM) induced repetitive stimulations of the transmembrane ion current (ΔI_{BzATP1} and ΔI_{BzATP2}). (*B*) BzATP-induced current change was examined in absence (ΔI_{BzATP1}) and presence (ΔI_{BzATP2}) of OLT1177 (OLT1177, 100 µM; N=8). (*C*) Normalized ΔI_{BzATP} values (ΔI_{BzATP2}/ ΔI_{BzATP1}) from experiments as shown in panel (*A*) and (*B*). Neither 100 µM nor 0.1 nM OLT1177 had an impact on ΔI_{BzATP2}/ ΔI_{BzATP1}. All ΔI_{BzATP2}/ ΔI_{BzATP1} are shown as individual data points, bars represent median, whiskers percentiles 25 and 75. Statistical analyses were performed using the Kruskal-Wallis followed by Mann-Whitney rank-sum test. (*D-F*) Representative current traces of water injected control oocytes (no expression of hP2X7 receptors). Neither repeated application of BzATP (10µM; N = 5), nor application of 100 µM OLT1177 (N = 4) or 0.1 nM OLT1177 (N = 4) alone and with BzATP induced any changes in transmembrane ion current.



SI Appendix Fig. S11. Pharmacokinetics after repeated dosing of OLT1177. Mean (± SD) plasma OLT1177 concentration versus time 24-hour profiles after a single dose (Day 1; solid lines) and after 8 daily doses (Day 8; dashed lines) of OLT1177 at 100 mg (red), 300 mg (blue) and 1000 mg (green).